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Research Paper

Assessment of the pathogenicity of cell-culture-adapted Newcastle disease virus strain Komarov

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Abstract

Newcastle disease vaccines *hitherto in vogue* are produced from embryonated chicken eggs. Egg-adapted mesogenic vaccines possess several drawbacks such as paralysis and mortality in 2-week-old chicks and reduced egg production in the egg-laying flock. Owing to these possible drawbacks, we attempted to reduce the vaccine virulence for safe vaccination by adapting the virus in a chicken embryo fibroblast cell culture (CEFCC) system. Eighteen passages were carried out by CEFCC, and the pathogenicity was assessed on the basis of the mean death time, intracerebral pathogenicity index, and intravenous pathogenicity index, at equal passage intervals. Although the reduction in virulence demonstrated with increasing passage levels in CEFCC was encouraging, 20% of the 2-week-old birds showed paralytic symptoms with the virus vaccine from the 18^{th} (final) passage. Thus, a tissue-culture-adapted vaccine would demand a few more passages by CEFCC in order to achieve a complete reduction in virulence for use as a safe and effective vaccine, especially among younger chicks. Moreover, it can be safely administered even to unprimed 8-week-old birds.

Key words: Newcastle disease, Komarov strain, chicken embryo fibroblast, Newcastle disease vaccine.

Introduction

Newcastle disease (ND) is a highly contagious viral disease of poultry that is caused by avian paramyxovirus serotype 1 (APMV 1), which together with viruses of other APMV serotypes (APMV 2-9) has been placed in the genus *Avulavirus*, subfamily *Paramyxovirinae*, and family *Paramyxoviridae* (Deleeuw and Peeters, 1999; Orsi *et al.*, 2010a).

The mesogenic Mukteswar strain was independently developed in India by G.S. Iyer through egg passage. Subsequently, the mesogenic Komarov strain was obtained by A. Komarov in Palestine, via serial passages of the virulent strain through ducklings (Czegledi *et al.*, 2003). The Komarov vaccine, also known as the Haifa vaccine strain (Mazia, 1990), is being used by most of the African countries and is less pathogenic than the Mukteswar strain (Grimes, 2002).

For several years now, embryonated chicken egg (ECE) adapted mesogenic live vaccines (strains K, R_2B, etc.) have been employed only among 8-week-old chicks that had already been primed at 2 weeks of age with a lentogenic vaccine such as strains LaSota, F, etc. (Reddy and Srinivasan, 1992; OIE, 2008). Otherwise, the mesogenic vaccine causes mortality in unprimed chicks, even at the age of 8 weeks. Mesogenic vaccines may predispose the poultry flock to viral and secondary bacterial infections (Bhaiyat *et al.*, 1994; Orajaka and Ezema, 2004; Saif *et al.*, 2008)

Owing to the drawback of mesogenic strains, the Commission of the European Communities (CEC) recommended against the use of vaccines containing an intra-

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cerebral pathogenicity index (ICPI) value of more than 0.4 (CEC, 1993). Since, all mesogenic vaccines have an ICPI value of more than 0.4, only lentogenic strain vaccines are permitted to be used. Available lentogenic strains are very mild in nature; thus, a lentogenic strain that produces not adequate immunity. Hence, it is required to reduce the virulence of the mesogenic strain to a lentogenic in such a way as to maintain an ICPI value close to 0.4 for producing a better immunity in birds. Keeping in view the above-mentioned drawbacks from the virulent nature of mesogenic strains and the CEC standards, an attempt was made to reduce the mesogenic virulence to a lentogenic virulence level by chicken embryo fibroblast cell culture (CEFCC). Pathogenicity trials of the tissue culture (TC)-passaged NDV strain K vaccine virus were conducted in vitro and in vivo, at various passage levels, to assess the extent of reduction in virulence, with the aim for it to be used in all age group of chicks, possibly without any post-vaccinal reactions.

Materials and Methods

NDV strain K vaccine

The freeze-dried RDV strain K vaccine virus was procured from the Institute of Veterinary Preventive Medicine, Raniphet, Tamil Nadu, India.

Embryonated chicken eggs

Embryonated chicken eggs were obtained from specific pathogen-free birds, which were maintained in the Department of Veterinary Microbiology, Veterinary College and Research Institute, Namakkal, India.

Birds

Day-old Babcock male chicks were procured from commercial hatcheries at Namakkal, India.

Preparation of “seed” virus for the first passage in chicken embryo fibroblast primary cell culture

One mL of freeze-dried vaccine virus was reconstituted and diluted to 1:1000 with phosphate-buffered saline, and then antibiotics were added at 200 IU of benzyl penicillin, 200 μg of streptomycin sulfate, and 200 IU of nystatin per milliliter of inoculum, to prevent bacterial and fungal contaminations. After the “processed” seed virus had been incubated at 37 °C for 30 min, it was inoculated into five 9-day-old ECEs. The eggs were then placed in an incubator containing a water tray in order to maintain the humidity at 60-65% with a set temperature of 38° C. Amnio-allantoic fluid (AAF) was harvested from the infected embryos, which had died after 24 h. The harvested AAF was centrifuged for 30 min at 3000 rpm at 4 °C and the supernatant was frozen at -20 °C as 2 mL aliquots. These aliquots were employed as seed virus for the first passage in CEFCC. The embryos were observed for embryopathy and specific lesions caused by the virus (Grimes, 2002).

Chicken embryo fibroblast primary cell culture

Ten-day-old actively moving embryos were used for the cell culture, and the rapid trypsinization procedure was carried out by employing 0.25% trypsin. The trypan blue dye exclusion technique was used for determining the viable cell count with the aid of a hemocytometer, and the cell concentration was adjusted to 2 x 10^6 cells/mL with growth medium. The cells were seeded in culture bottles, incubated at 37 °C, and then observed for confluent monolayers (Freshney, 2010).

Passaging of virus in CEFCC

Culture flasks with an 80% monolayer were inoculated with 1 mL of undiluted embryo-propagated virus (*i.e.*, AAF). After a 60-90 min adsorption time at 37 °C, the monolayer was washed with maintenance medium and then covered with the same medium. The occurrence of any cytopathic effect (CPE) was observed at 8 h intervals (Freshney, 2010).

Infection of subsequent passages

The “start-up” virus of each passage was subjected to three freeze-thaw cycles, followed by a light clarifying centrifugation to remove cell debris, and the supernatant was used for further passages. In the case of the serial blind passages, the undiluted inoculum was used as the seed virus. For active passages, a 1:100 dilution of previous passages was used as the seed material. Production of about 80% CPE was taken to be the harvesting criterion. The culture fluids with maximum CPE were preserved at -20 °C and used as the inoculum for the second passage. Similarly, the cell culture fluid of the previous passage was used for subsequent passages, up to the 18^{th} passage level (Schat and Purchase, 1989).

Passages in CEFCC

In total, 18 passages could be carried out with the CEFCC system. Although visible CPEs could already be noticed after four blind passages, the infected TC fluids of all blind passages were nonetheless confirmed for the presence of virus by inoculation in ECEs. Similar recordings were made in the case of 14 “active” passages, along with observation of specific CPE. A microtitration technique with a 10-fold dilution was used to assess the $TCID_{50}$ value for alternative passages (Schat and Purchase, 1989). Micro-hemagglutination (HA) tests were also conducted with the seed and all the cell-culture-passaged viruses (OIE, 2008).

Modified spot or rapid HA test

The spot HA test is a rapid test used to identify the presence of a virus in the egg/TC bottle. One milliliter of the AAF or 1 mL of TC fluid was added to 1 mL of 33.3% chicken red blood cells (CRBC) held in a white porcelain plate. Both the CRBC and test samples were properly mixed and observed for HA within 1 min (Grimes, 2002; Ullah *et al.*, 2004).

Pathogenicity indices

The extent of reduction in virulence of the NDVK (mesogenic) strain was assessed on the basis of pathogenicity indices; namely, mean death time (MDT) in 9-day-old embryos, intracerebral pathogenicity index (ICPI) in day-old chicks, and intravenous pathogenicity index (IVPI) in 6-week-old chicks, conducted using seed virus and TC-passaged viruses (5^{th}, 10^{th}, 15^{th}, and 18^{th} passages). A reduction in the virulence of the TC-adapted virus was further confirmed by inoculating it into 2-week-old and 8-week-old chicks (OIE, 2008).

Results

The revived freeze-dried egg-adapted vaccine virus, which served as seed material for passage in the TC system, possessed high HA (2^{10}) and EID_{50} ($10^{7.5}$) titers. The potency of the seed virus was further confirmed by the appearance of specific lesions in the ECEs; namely, occipital and pedal hemorrhages (Figure 1). No significant (cytopathic) changes were noticed in the CEFCC system for up to three passages. However, the virus presence was confirmed by the observation of specific lesions in the ECEs. Adaptation of the virus in CEFCC was observed (CPE initiation) from the 4^{th} passage onwards, and the extent of the CPE increased with progressive passages upto the 18^{th}/final passage of this study (Figures 2A and 2B)

Figure 1 - Specific lesions: Occipital and pedal hemorrhages. (A) Seed-virus-infected embryo showing typical occipital hemorrhage. (B) Passaged (5^{th}) virus-inoculated embryo showing mild occipital hemorrhage. (C) Uninoculated control without any occipital hemorrhage.

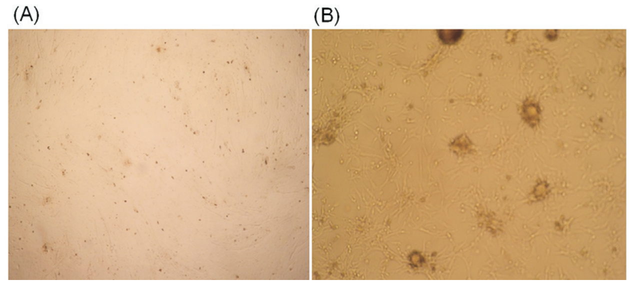

Figure 2 - (A) Uninfected chicken embryo fibroblast (CEF) monolayer. (B) Infected chicken embryo fibroblast monolayer showing clumping, rounding, and syncitial formations of the CEF cells.

The $TCID_{50}$ titers of active passages increased from $10^{3.4}$/mL (4^{th} passage) to $10^{5.3}$/mL (18^{th} passage). Similarly, the EID_{50} titers of the TC-passaged viruses also increased from $10^{3.4}$/mL (2^{nd} passage) to $10^{6.2}$/mL (18^{th} passage) (Figure 3).

The MDT increased from 71.4 h (5^{th} passage) to 92.1 h (18^{th}/final passage). The ICPI value decreased by 0.1 in the case of the 5^{th} passage (1.2) compared with the seed virus (1.3), and the value decreased further by 0.4 in the case of the 18^{th} passage (0.8) relative to the 5^{th} passage (1.2). The IVPI values of the seed and the selected TC-passaged viruses (5^{th}, 10^{th}, 15^{th}, and 18^{th} passages) were all zero (Table 1).

Discussion

The NDVK seed virus demanded a few blind passages for adaptation in CEFCC. Similarly, Parimal and Padmanaban (1990) reported that the NDV Komarov strain adapted in CEFCC after four blind passages. However, Padmaraj *et al.* (1991) recorded two blind passages for NDVK in the BHK_{21} (Razi) cell line. After adaptation of the NDVK virus in CEFCC, lesions appeared after 48 h, and more pronounced CPEs were observed from the 7^{th} passage onwards; later, the duration for CPE occurrence was reported to decrease from 48 h post-inoculation (p.i.; 5^{th} passage) to 24 h p.i. (8^{th} passage) (Padmaraj *et al.*, 1991). Similarly, Joshi *et al.* (2002) observed cell rounding

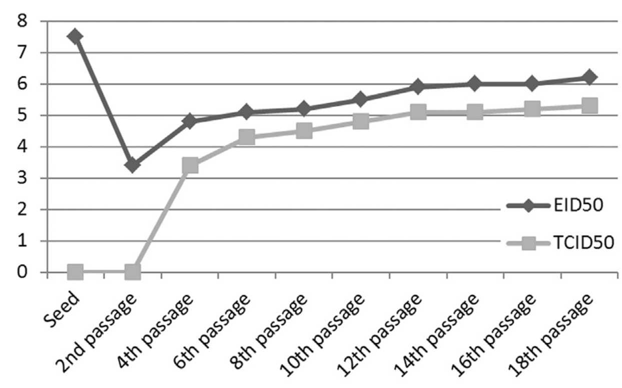

Figure 3 - EID_{50} and $TCID_{50}$ titers of the seed and TC-passaged viruses.

Table 1 - Pathogenicity indices and number of birds showing symptoms with Newcastle disease virus strain K (NDVK) seed and tissue-culture-passaged viruses.

		Seed (NDVK)	5th Passage	10th Passage	15th Passage	18th Passage
Pathogenicity indices	MDT (h)	64	71.4	78.6	86.5	92.1
	ICPI	1.3	1.2	1.1	0.9	0.8
	IVPI	0.0	0.0	0.0	0.0	0.0
Percentage of infected birds	Two weeks	80%	70%	50%	40%	20%
	Eight weeks	10%	10%	0%	0%	0%

MDT: mean death time; ICPI: intra-cerebral pathogenicity index; IVPI, intravenous pathogenicity index.

and clumping as early as 48 h p.i., and cellular degeneration within 96 h p.i.

The MDT values were increased and the ICPI values were decreased with our TC-passaged virus, which clearly indicates the difficulty of the egg-adapted virus in tissue culture. The reason could be attributed to the virus unable to replicate in the cell culture, owing to the absence of specific receptors, which leads to faulty production of the viral protein (Arora *et al.*, 2003). The progress of the infectivity ($TCID_{50}$ and EID_{50}) was also in good accordance with the observations of Bansal and Kumar (1975).

OIE (2008) has established an ND virus to be a lentogenic strain if it has an MDT of more than 90 h and an ICPI value of less than 0.5. Thus, based on these criteria, the final passage of the virus (*i.e.*, 18th passage) in our study fulfills only the MDT criterion, since it was more than 90 h (*i.e.*, 92 h), but the ICPI value was more than 0.5 (*i.e.*, 0.8). Oris *et al.* (2010b) characterized the isolated virus on the basis of the ICPI value alone and found it to be more important than the MDT value in establishing it as apathogenic strain. Hence, to meet the criteria of the Office International des Epizooties (OIE, 2008), the ICPI value of the virus needs to be reduced to 0.4. Thus, a few more passages are warranted to reduce the virulence to the lentogenic level.

The increase in MDT and decrease in ICPI values of the TC-passaged virus relative to the seed virus confirms the reduction in virulence of the seed or egg-adapted virus, when passaged in CEFCC. However, the reason for the IVPI value remaining as zero from the seed virus upto the final passage remains same is unexplainable; because, the "start-up" virus itself has a value of zero. Nevertheless, it at least indirectly indicates no increase in virulence.

In unprimed 8-week-old birds, 10% of the birds exhibited respiratory symptoms with the seed, and 5th and 10th passage viruses. However, the 15th and 18th passage viruses did not produce any specific symptoms. These results confirm that the Komarov vaccine causes respiratory symptoms in unprimed chicks, which correlates with the results of previous studies (Reddy and Srinivasan, 1992; OIE, 2008)

In 2-week-old chicks, the 5th passage virus elicited ND symptoms similar to the seed virus. The 18th passage virus elicited ND symptoms among 20% of the birds of this age, with lower intensity. The substantive reduction in number of chicks evincing ND symptoms with the 18th passage virus, with significant reduction in the intensity of the symptoms, clearly indicates a marked reduction in the virulence of the virus, when compared with the seed virus.

Elamin *et al.* (1993) experimentally verified that the nasal route of vaccination also gives similar immunity as wing-web method. This would facilitate our research, because the passaged Komarov strain could be safely and easily administered through the nasal route as with the lentogenic vaccine.

Conclusions

In conclusion, when NDVK chick-embryo-adapted vaccine viruses were passaged 18 times in the CEFCC system, the $TCID_{50}$ values of the passaged viruses in TC and embryonating eggs are increased, indicating an increase in total infective particles and thus confirming the progressive adaptation of the virus in the CEFCC system. The values of the pathogenicity indices for the passaged viruses were altered towards those of lentogenic strains. However, the "actual" lentogenic values could not be reached. Hence, a few more passages are warranted to render the strain more lentogenic in nature for the safe use in 2-week-old chicks in order to give a better protective titer compared with other commercially available lentogenic vaccines.

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